ACTIVATED SLUDGE AS A SOURCE OF VITAMIN B_{12} FOR ANIMAL FEEDS *

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In the course of studies of aerobic oxidation of dairy wastes, the presence of vitamin B_{12} in activated sludge was suspected because of the microbial nature of the floc and the known synthesis of this vitamin by intestinal and soil bacteria. This report presents results of a study on the vitamin in activated sludge from municipal sewage treatment plants and indicates the possible utility of activated sludge for fortifying animal feeds with this vitamin. The discovery of vitamin B_{12} in activated sludge has been reported in a preliminary note (5).

Vitamin B_{12}, the anti-pernicious anemia factor of liver, was isolated almost simultaneously in this country and in England in 1948. Clinical tests have shown that it is effective in various anemias, in cirrhosis of the liver, and in other diseases, and nutritional studies have indicated that it stimulates growth and appetite in children. As has been the case with other vitamins, full evaluation of the effects of vitamin B_{12} in humans will require biochemical and clinical study for many years.

This vitamin also has noteworthy effects in the nutrition of domestic animals (11). The isolated product markedly improves the utilization of vegetable protein by chickens and swine, producing up to a 20 per cent greater weight increase per pound of food consumed. The optimum increase is obtained by the addition of only 10 to 15 mg. of vitamin B_{12} per ton of feed. The resultant decrease in amount of grain required constitutes a substantial reduction in the cost of production of meat. This effect is closely related to that of the "animal protein factor" (APF), which has been studied by animal nutritionists for a number of years. This factor, essential for the growth of chicks and swine, has been found in fish meal, fish solubles, liver, and a wide variety of other animal products. Tests have shown that vitamin B_{12} is the primary factor in APF, and the latter term has been largely abandoned in recent months.

The rumen contents of ruminants and their feces contain appreciable quantities of vitamin B_{12}, which is apparently synthesized by the microbial flora present. A number of soil microorganisms perform this synthesis, and commercial production of vitamin B_{12} concentrates by bacterial fermentation is well established. The vitamin is required for the growth of other bacteria, notably certain *lactobacilli,* a fact which has been utilized in the development of microbiological assay procedures.

Intensive study of the chemistry of vitamin B_{12} has shown that it can be isolated as crystalline red needles (melting point 210° to 220° C.), which have the approximate composition $C_{61\text{-}64}\ H_{86\text{-}92}\ N_{14}O_{13}\ PCo$. There are at least two forms of the vitamin. By catalytic hydrogenation, a crystalline substance, vitamin B_{12a}, is produced. This product has a lower biochemical activity than vitamin B_{12}, and the ratio of the activities differs, depend-

* Report of a study made under the Research and Marketing Act of 1946.

ing on the animal or bacterial species used in the test. The existence of other biologically active forms of the vitamin is suspected, so that at present the most satisfactory designation for the active factors in a tissue or crude extract is "vitamin B_{12} complex." In this paper, the term "vitamin B_{12}" is used for convenience to mean "vitamin B_{12} complex."

Assay of Vitamin B_{12}

Analysis of animal and plant extracts for vitamin B_{12} by microbiological assay is difficult, for the test organisms require a relatively complete mixture of amino acids, vitamins, and minerals. Moreover, the vitamin B_{12} requirements of the organism can be met, at least in part, by other constituents of the extracts. Extensive study has shown that most of these compounds which give falsely high values are stable to treatment with alkali (0.2 N NaOH for 30 min. at 100° C.), whereas vitamin B_{12} is destroyed by such treatment. The direct assay is, therefore, often "corrected" by subtracting this nonspecific activity. In this report, the direct assay (9) is called Method I, and this value less the nonspecific alkali-stable activity (4) is called Method II.* The growth of the test organism, *Lactobacillus leichmannii* ATCC 4797, is measured by turbidimetric analysis after 24 hr. at 37° C. A standard vitamin B_{12} activity curve is run in parallel with every series of determinations. Method II, with minor modifications, is the procedure commonly used for assaying the vitamin in feeds and feed supplements.

A more complicated but specific assay for vitamin B_{12} has been developed in which the true vitamin is first separated from those compounds which give the nonspecific growth response (10). This separation is made by paper chromatography, a relatively new technique utilizing the differential rate of migration of the solvent and of the various dissolved constituents as a solution travels along a sheet of filter paper by a wicklike action. The portion of the paper containing the vitamin B_{12} is cut out, and the amount present is then determined by direct growth assay. This procedure is designated Method III. It is believed to give the most conservative estimate of the vitamin B_{12} content of extracts that can be obtained by microbiological assay. Tests for the nonspecific activity of the extracts are made by measuring the growth response caused by the other constituents, which have been separated from the vitamin B_{12} complex by differential migration on the paper.

The ultimate evalution of vitamin B_{12} potency of feeds and feed supplements is its effect on animal growth. Chick-growth tests have been developed which are relatively satisfactory. It has been well demonstrated that the availability of vitamin B_{12} in microbiological assays may differ markedly from its availability to chickens and swine. The digestive system of animals apparently can release the vitamin from some types of chemical combination in which it is unavailable to microorganisms. Moreover, the test organisms can convert some precursors to the vitamin which the higher animals cannot.

Assay of Commercial Sludge

Samples of heat-dried activated sludge now on the market were assayed for their vitamin B_{12} content (Table I). Milorganite was purchased on the

* The vitamin is extracted from the sludge by suspending it in acetate buffer and heating it at 100° C. in an autoclave for 30 min. The buffer used contains 207 g. sodium acetate and 126 g. acetic acid per liter, pH 4.5. A distilled-water extract of dried commercial sludge prepared similarly has a pH of 4.1 to 4.5, and the amount of vitamin B_{12} extracted by water is equal to that extracted by the buffer solution. However, use of buffer solutions for extraction of vitamin B_{12} from miscellaneous plant and animal tissue is preferable.

TABLE I.—Vitamin B_{12} Content of Commercial Dried Sludge[1]

Source	Moisture (%)	Vitamin B_{12} Determined (μg./g.)			
		Method I[2]	Non-spec. Activity	Method II[3]	Method III[4]
Milwaukee, Wis. (Milorganite)	10.8	4.11	0.17	3.9	4.5
Chicago, Ill. (Southwest plant)	4.6	3.74	0.30	3.4	2.9
Chicago, Ill. (Calumet plant)	2.2	1.81	0.19	1.6	1.6
Houston, Tex. (Hou-Actinite)	10.0	3.77	0.22	3.5	3.4

[1] Calculated on samples as received.
[2] Direct growth assay with *Lactobacillus leichmannii*.
[3] Total indicated by Method I less the non-specific activity shown by growth of *L. leichmannii* after vitamin B_{12} had been destroyed by alkali treatment.
[4] Growth assay with *L. leichmannii* after vitamin B_{12} had been separated from other growth stimulants by paper chromatography.

open market; the other three samples were furnished by the Chicago (Ill.) Sanitary District and the Houston, Tex., sewage treatment plant. Moisture determinations were made in the laboratory, and the vitamin B_{12} contents were determined by the three methods just described. The differences between three of the four samples may or may not be significant. The product from the Chicago Calumet plant differed in physical properties and in vitamin B_{12} content. The vitamin is sensitive to heat and previous data have shown that there is marked loss during drying. Therefore, the amount present in the dried product does not necessarily reflect the amount present initially, but is undoubtedly related to the method of coagulating and drying employed. Further investigation of the loss during the processing of the sludge should be made if it is to be marketed as a feed supplement.

The vitamin B_{12} content of these products must be considered in relation to the nutritional requirements of animals. An adequate supply of this vitamin has been estimated as 10 to 15 mg. per ton of feed (7). A vitamin B_{12} content of 1.5 mg. per lb. (3.3 μg. per g.) has been proposed by the Association of American Feed Control Officials as the required level for a commercial feed supplement. Thus, 7 to 10 lb. of a minimum potency supplement would suffice for a ton of feed. Three of the samples tested showed such a content when determined by Method II, which is generally used in the industry. The values obtained by Method III are comparable with those of Method II.

Method III gave further information because a measure of the non-specific activity was obtained. The results (not tabulated) show that there was little non-specific activity in the four samples. The quantity found was about the same as that indicated by the alkaline destruction test.

Distribution in Activated Sludge System

Inasmuch as vitamin B_{12} is synthesized by various microorganisms, a short study was made of the source of the vitamin in the sludge.* The data in Table II were obtained by assay Method II. After preliminary experiments lasting for several weeks, a series of runs was made on 5 successive days of relatively stable operation. A daily composite sample was obtained by taking a grab sample each 4 hr. proportional to the flow of the previous

* These results were obtained through the excellent cooperation of R. M. Bolenius, superintendent, Abington Township sewage treatment plant, Montgomery County, Pa. The design of this plant has been described previously (2).

TABLE II.—Vitamin B_{12} in a Municipal Activated Sludge Plant

Characteristic	Date: April, 1951					
	11	12	13	14	15	Av.
Primary effluent:						
Flow (m.g.d.)[1]	1.407	1.372	1.360	1.348	1.345	1.366
Total solids (g./l.)[1]	0.29	0.36	0.30	0.29	0.29	0.31
Vitamin B_{12} (μg./l.)[1]	0.12	0.34	1.12	0.28	0.46	0.46
Vitamin B_{12} (mg./kg. solids)	0.41	0.94	3.73	0.97	1.59	1.52
Vitamin B_{12} (g./day)	0.64	1.77	5.77	1.43	2.34	2.39
Aerator influent:						
Return sludge (m.g.d.)[1]	0.575	0.539	0.531	0.531	0.551	0.545
Total flow (m.g.d.)[1]	1.982	1.911	1.891	1.879	1.874	1.907
Total solids (g./l.)[1]	2.9	3.5	3.2	2.9	3.3	3.2
Vitamin B_{12} (μg./l.)[1]	42.9	43.7	41.7	43.6	45.7	43.5
Vitamin B_{12} (mg./kg. solids)	14.8	12.5	13.0	15.0	13.8	13.6
Aerator effluent:						
Total solids (g./l.)[1]	2.1	2.1	2.1	2.1	2.4	2.2
Vitamin B_{12} (μg./l.)[1]	40.8	38.7	43.7	44.2	41.8	41.8
Vitamin B_{12} (mg./kg. solids)	19.4	18.4	20.8	21.0	17.4	19.4
Waste sludge:						
Flow (g.p.d.)[1]	5130.	5330.	6850.	8210.	5000.	6104.
Total solids (g./l.)[2]	9.3	11.5	10.6	9.5	11.2	10.4
Vitamin B_{12} (μg./l.)[2]	148.	154.	146.	154.	154.	151.
Vitamin B_{12} (mg./kg. solids)	15.9	13.4	13.8	16.2	13.8	14.6
Vitamin B_{12} (g./day)	2.86	3.11	3.78	4.47	2.92	3.49

[1] Determined values.
[2] Values calculated from return sludge flow, primary effluent, and aerator influent.

4-hr. period. Negligible rain fell during the test period. The total sewage flow was constant, as was the solids content of the effluent from the primary settling tank. A rather surprising variation in the vitamin B_{12} content of this effluent was observed. A similar variability of the primary effluent was also observed in the preliminary tests.

An attempt was made to test for synthesis in the aerator by analyzing the aerator influent and the effluent. Because of the oxidation of organic matter, an increase of vitamin B_{12} on a solids basis was observed. However, there was no measurable change on a liquid volume basis.

The vitamin B_{12} content of the return sludge, and therefore of the waste sludge, can be determined by calculation from the primary effluent and the aerator influent. In the tests, an average daily output of 3.49 g. of vitamin B_{12} *versus* an intake of 2.39 was observed. The results obtained on the aerator effluent for the final day indicate that there was at least as much total solids and as high a vitamin B_{12} content in the system at the end of the experiment as there was initially. These data, therefore, suggest some synthesis of the vitamin in the activated sludge process. Fluctuations from day to day were so great that it was not possible to make a quantitative estimate of the proportion of the vitamin synthesized without extensive experiments.

The primary effluent, however, contained an appreciable amount of the vitamin, for the low content per unit volume must be multiplied by the total flow of about 1.4 m.g.d. The excess sludge is wasted through the primary settling tank in this plant. Therefore,

a portion of the vitamin might be considered as coming from this source. But a separate run of 3 days in which no sludge was wasted gave no indication that the B_{12} content of the primary effluent arose from this source, for when no waste sludge was introduced into the primary tank, the vitamin B_{12} content of the effluent was at least as high as when sludge was being wasted. These preliminary results can be interpreted, therefore, as indicating that the vitamin probably is synthesized in the system, but more especially that the aeration tank acts as a refining and concentrating system. The incoming sewage contained about 0.5μg. per l.; the waste sludge contained about 150 μg. per l. The corresponding values on a total solid basis are 1.5μg. per g. and 15 μg. per g., a 10-fold increase. Obviously, any vitamin B_{12} present in the plant effluent is ignored in these calculations. The excellent operation of the plant and the low B.O.D. of the effluent justify such a procedure in a preliminary study of this kind.

Cobalt Content

Vitamin B_{12} contains 4.4 per cent cobalt as an essential constituent. This element is present only in traces in surface waters and in plant and animal tissues. It was, therefore, of interest to determine the cobalt content of the dried sludge. Commercial sludge contains about 35 per cent of sand and clay, which have an appreciably higher content of cobalt than plant tissue (3). Also, the large amount of iron in the commercial sludge interfered with an accurate analysis for total cobalt. Therefore, the cobalt extractable by hot water (see vitamin B_{12} assay procedure) was determined as a measure of the available cobalt content. The analytical procedure used was that of Deijs and Feldmeyer (3), except that readings were made on a Coleman spectrophotometer at 500 mμ, as recommended by Ovenston and Parker (6).

Table III shows that much more cobalt is extractable by hot water than is accounted for by the vitamin B_{12} content. The first interpretation of these results would be that cobalt is not limiting in the synthesis of the vitamin. The actual concentration of available cobalt, however, is low. The extractable cobalt of the dry solids is approximately 1,000 μg. per kg., or 1 p.p.m., a portion of which may be obtained from the inorganic sand and clay. Assuming that the living microorganisms in the floc contain 75 per cent moisture, the cobalt available within the cells for vitamin B_{12} synthesis and any other possible requirement would be 0.25 p.p.m. of living cells. Addition of small amounts of cobalt, say 0.01 to 0.10 p.p.m. to the aerator influent might result in a significant increase in vitamin B_{12} content of the dried sludge. Experiments of this type are planned in laboratory studies on aeration of milk waste. Such tests in municipal treatment plants would appear to be of great interest.

The amount of sludge solids extracted by the hot-water extraction procedure was determined, as such an extraction might be feasible for the preparation of more potent vitamin B_{12} concentrates. About 4 per cent of the organic matter (8) and a similar amount of inorganic solids were extracted (Table IV). The vitamin B_{12} content of this extract, calculated on a total solids basis, was, therefore, 10 to 15 times as great as in the dried sludge itself. No experiments have been made on recov-

TABLE III.—Cobalt Content of Sludge

Source	Cobalt Content (μg./kg.)	
	Calc. from B_{12} Content	Extr. by Hot Water
	μg./kg.	μg./kg.
Milwaukee	170	1070
Chicago, Southwest	150	510
Chicago, Calumet	60	590
Houston	150	800

TABLE IV.—Analysis of Hot-Water Extract of Sludge[1]

Source	Org. Sol.[2] (%)	Tot. Sol.[3] (%)
Milwaukee	4.6	8.4
Chicago, Southwest	4.5	8.2
Chicago, Calumet	3.2	6.3
Houston	3.4	6.6

[1] 25-gm. samples in 100 ml. water were heated in the autoclave for 30 min. at 100° C. The suspension was cooled, diluted to 250 ml., and centrifuged for 30 min. at a RCF of 2,500 g. Results are calculated on the original air-dry weight.

[2] By oxygen consumed.

[3] By weight.

ery of vitamin B_{12} after drying this extract.

Chick-Growth Assays

Chick-growth assays were conducted by R. J. Lillie and H. R. Bird of the Bureau of Animal Industry, U. S. Department of Agriculture, at Beltsville, Md. Milorganite was the only commercial sludge tested. Several assays of one sample of this product for vitamin B_{12} gave values of about 2.0 μg. per g.* Dr. Bird has given the information that fermentation products usually show a higher content of vitamin B_{12} by microbiological determination than by chick-growth test. This anomaly will probably be resolved in the near future. At present the microbiological test, essentially according to Method II, is the accepted assay procedure and the one on which the proposed standard of 1.5 mg. per lb. is based.

Economic Consideration

Dried activated sludge for fertilizer use is sold at the plant at about 1¢ per pound. A vitamin B_{12} content of 3.3 μg. per g., or 1.5 mg. per lb., is the required level proposed by the Association of American Feed Control Officials. If a ton of feed were supplemented with 15 mg. of vitamin B_{12}, addition of 10 lb. of activated sludge would suffice. This cannot be considered as a cost of no more than 10¢ per ton of feed for supplementation; costs of assay and shipping would undoubtedly increase this figure. However, the present cost of vitamin B_{12} concentrates produced by fermentation is estimated to be about 40¢ per ton, so there would appear to be a satisfactory economic basis for use of activated sludge in feeds if regulatory agencies rule its use acceptable.

* These results will be published as part of a larger study by the Bureau of Animal Industry group.

Discussion

The striking result of these experiments is the presence of vitamin B_{12} in significant amounts in commercial dried sludge. Many unanswered questions are pertinent to the further consideration of this result. As this laboratory is not in a position to carry out further research on municipal sludge, the major points are presented for the consideration of municipal sewage authorities.

1. If dried activated sludge is to be incorporated directly into feeds, its use must be approved by the U. S. Food and Drug Administration and the respective State Feed Control officials.

2. The possibility of preparing richer concentrates by extraction of the vitamin must be considered. The dry residue remaining after extraction would still have approximately the same content of major constituents as the original dry material and presumably would be comparable in fertilizer value.

3. Enrichment of the vitamin B_{12} content by addition of cobalt to the aerators is a possibility. Addition of 0.01 to 0.10 p.p.m. would increase the amount available to the cells, probably without significantly raising the cobalt content of the final plant effluent. There is no direct evidence that a consequent increase in vitamin B_{12} would result, but such addition is the usual practice in commercial fermentation for production of this vitamin.

4. Loss of vitamin B_{12} in commercial drying of sludge is about 60 to 75 per cent. No published figures for the loss in drying fermentation vitamin B_{12} concentrates are available, but it has been repeatedly stated that appreciable loss in potency occurs. Determination should be made of drying conditions which are economically feasible and yet result in minimum loss of the vitamin. It has been established that greater destruction is found by microbiological tests than by animal feeding tests. Inasmuch as the potential use is in animal feeding, the latter assay should be used in studies of this destruction.

5. The chick-growth tests were short-time assays (2 weeks) on young chicks. Vitamin B_{12} response was shown by an increased rate of growth over that of control chicks. In these tests, the positive response proved that no toxicity resulted from incorporation of dried sludge in the diet. However, experiments over a longer period and with other animals should be conducted to prove conclusively the lack of toxicity of this material in the low levels required. Some years ago, Bohstedt conducted swine feeding experiments in which the use of Milorganite as a major source of protein in the diet was attempted. At high levels, definite toxicity was indicated (1). These experiments were never reported in detail.

Summary

Activated sludge contains significant amounts of vitamin B_{12}; the dried commercial fertilizer has a content of approximately 3.5 to 4 mg. per kg. This amount is comparable with that required in commercial animal feed supplements (3.3 mg. per kg.)

A study of the distribution of this vitamin in activated sludge from municipal sewage treatment plants indicated that part of it is derived from the raw sewage and part synthesized by microbiological action in the aeration tanks.

A number of possible extensions of this study are pointed out.

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